2910-Pos Board B680
Voltage Dependent Anion Channel-3 (VDAC3) is the Major Isoform Contributing to Mitochondrial Metabolism in HepG2 Cells and is Regulated by Free Tubulin and Erastin
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BACKGROUND: VDAC controls the flux of hydrophilic metabolites into mitochondria. *In vitro,* tubulin closes VDAC and *in situ* free tubulin dynamically modulates $\Delta\Psi$. Erastin, a ligand for VDAC, increases $\Delta\Psi$ in HepG2 cells. Here, we hypothesize that VDAC regulates mitochondrial metabolism and that free tubulin decreases and erastin increases VDAC conductance. Our **AIM** was to determine $\Delta\Psi$, ATP, NADH, and response to free tubulin and erastin after VDAC knockdown (KD). **METHODS:** HepG2 cells were transfected with siRNAs (5 nM, Ambion) against VDAC1/2/3. At 48 h after transfection, $\Delta\Psi$ was assessed by fluorescence of tetramethylrhodamine methylester (TMRM) and NADH by autofluorescence using confocal/multiphoton microscopy. Fluorescent beads were fiduciary markers. Adenine nucleotides were determined by HPLC. **RESULTS:** siRNA decreased mRNA and protein for each VDAC isoform by ~90%. Double KD of VDAC1/2, VDAC1/3 and VDAC2/3 decreased TMRM fluorescence by ~20, 55 and 73%, respectively, compared to 100% for non-target siRNA. In reconstituted bilayers, VDAC from HepG2 formed typical anion selective and voltage-gated channels reversibly blocked by dimeric tubulin. Nocodazole decreased $\Delta\Psi$ by ~61% in non-target cells and 43, 14 and 17% after KD of VDAC1/2, VDAC1/3 and VDAC2/3. VDAC3 KD decreased ATP by ~48%, total adenine nucleotides by ~45%, NADH by ~33% and the NADH/NAD ratio by ~60%. Erastin increased $\Delta\Psi$ by ~46-48% in non-target and VDAC1/2 KD cells but failed to return $\Delta\Psi$ to baseline levels after VDAC1/3 and VDAC2/3 KD. Erastin also blocked and reversed depolarization induced by nocodazole. **CONCLUSION:** VDAC3, the least abundant isoform, contributes most to maintenance of mitochondrial metabolism in HepG2 cells. Erastin antagonizes the inhibitory effect of free tubulin. These results indicate that VDAC, especially VDAC3, regulates mitochondrial metabolism in hepatoma cells.

2911-Pos Board B681
Nrf2 Controls Mitochondrial Bioenergetics
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The transcription factor nuclear factor E2-related factor 2 (Nrf2) and its repressor Kelch-like ECH-associated protein 1 (Keap1) are known to regulate the antioxidant response element (ARE) pathway responsible for controlling the expression of a network of cytoprotective genes, including antioxidant and anti-inflammatory genes as well as genes involved in mitochondrial biogenesis. Using biochemical techniques in addition to live cell imaging and respirometry we now show that the Keap1-Nrf2 pathway is further involved in the control of mitochondrial metabolism. Experiments in mouse embryonic fibroblasts from wild-type, Nrf2 and Keap1 knockout (KO) animals show that, compared to wild-type, cells lacking Nrf2 have decreased mitochondrial membrane potential while Keap1 KO cells on the other hand show an increase. Interestingly, Nrf2 KO cells show significant inhibition of the rate of oxygen consumption, suggesting that the decrease in mitochondrial membrane potential is not due to mitochondrial uncoupling. We further demonstrate in primary neuroglia cultures (isolated from wild-type, Nrf2 KO and Keap1 knockdown mice) that this pathway has a significant effect on the FAD and NADH redox states of the cells and the defect could not be reversed by application of mitochondrial substrates. Finally, Nrf2 KO cells show increased dependence on glycolysis, as seen by both live cell imaging and western blot. In conclusion, the Keap1-Nrf2 pathway is not only important in the anti-oxidant defence of the cells but also plays a major role in energy metabolism.

2912-Pos Board B682
A Surface Plasmon Resonance-Based Two-Dimensional Screen for Protein Kinase Substrates Identifies Fumarase as AMPK Target
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This study describes a novel in vitro two-dimensional screening approach for kinase substrates that combines a biophysical interaction assay based on surface plasmon resonance and a standard in vitro phosphorylation assay. The interaction assay was optimized with appropriate prepurification procedures on a Biacore instrument. It selects for substrates interacting with a specific kinase, and can thus identify substrates that are preferentially phosphorylated e.g. by a specific kinase isoform. This approach was applied to isoforms of the heterotrimeric AMP-activated protein kinase (AMPK). AMPK is an emerging central cellular signaling hub in energy homeostasis and proliferation, but its signaling network is still incompletely understood. Using soluble rat liver proteins and full-length AMPK $\alpha 2$-$\beta 2$-$\gamma 1$ complex, several putative AMPK substrates were identified by mass spectrometry. One of them, fumarate hydratase (fumarase), was confirmed as an in vitro AMPK target which preferentially interacted with and was phosphorylated by the AMPK$\alpha 2$ isoform as shown by yeast-two-hybrid and in vitro phosphorylation assays. AMPK-mediated phosphorylation of fumarate hydratase led to significant activation of enzymatic activity in vitro and in vivo, suggesting that it is a bona fide AMPK substrate. This may have different physiological consequences, since the enzyme has a dual localization in the mitochondrial matrix and the cytosol. [AK and CP contributed equally to this work]

Electron & Proton Transfer

2913-Pos Board B683
Negative Cooperativity in the Reduction of Excitonically Interacting B-Hemes of the Cytochrome B6F Complex
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Cytochrome b6f and bc1 (bc) complexes, which provide the central charge transfer complex in respiratory and photosynthetic electron transport chains, are symmetric dimeric structures.1 A trans-membrane electron transfer pathway between hemes bp and bn2 exists in each monomer. Based on inter-heme distances this intra-monomer pathway (bp-bn) is preferred over an inter-monomer cross-over pathway between hemes bp1. Cross-over has been reported,3,4 although the branching ratio is not known. Previous studies on ferredoxin-dependent reduction of b6f by NADPH showed only half of the b-heme reduced in thylakoid membranes,5 and biphasic reduction with isolated complex.6 It was inferred that the rapid reduction occurred through intra-monomer electron transfer and that the split circular dichroism spectrum7,8 (node of the CD spectrum in the Soret band coincides with the heme absorbance maximum), implies that the hemes bp and bn interact excitonically in the monomeric unit. Here, monophasic chemical (dithionite) reduction of monomeric b6f complex, characterized by size-exclusion chromatography, proceeds 10-20 times more rapidly than in the dimeric complex. The reduced monomer showed full amplitude of the Soret band split CD spectrum. These measurements are consistent with ''half-sites reactivity'' observed for b-heme reduction in the yeast bc1 complex,9,10 in which reduction of one monomer is associated with a reduced rate of electron transfer to the second monomer of the bc1 dimer. The mechanism for this negative cooperativity could arise from a larger reorganization energy11 for inter-monomer electron transfer, or constraints imposed by the Rieske protein in the dimer. NIH-GM38323.1Cramer et al. 2011; 2Yamashita et al. 2007; 3Lanciano et al. 2010; 4Swierczek et al. 2010; 5Furbacher et al. 1989; 6Hasan et al., 2011; 7Palmer and Degli-Esposti 1994; 8Schoepp et al. 2000; 9Covian and Trumpower, 2008; 10Castellani et al. 2010; 11Marcus and Sutin 1985.

2914-Pos Board B684
Energy Transduction by Bacterial Complexes I
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Complex I (CpI) of respiratory chains is an energy transducing enzyme present in most bacteria and in all mitochondria. It is still the least understood complex of these chains, in spite of the structural data recently available[1-3]. This complex catalyses NADH:quinone oxidoreduction, coupled to ion translocation across the membrane.

The nature of the coupling charge of *Rhodothermus marinus* CpI was investigated using inside-out membrane vesicles. We observed that this CpI is able of H^+ and Na^+ transport, although to opposite directions. The H^+ is imported and Na^+ is exported from the vesicles, indicating that the H^+ is the coupling ion of the system [4]. The Na^+ transport is specific of CpI activity, being sensitive to its inhibitor rotenone, and stimulated by the presence of CCCP, a protonophore. We observed that although neither the catalytic reaction nor the establishment of the ΔpH requires the presence of Na^+, its presence increased the H^+-transport. We proposed a model for the coupling mechanism of CpI, suggesting the presence of two different energy coupling-sites, one working as a H^+-pump (Na^+ independent), and the other functioning as a Na^+/H^+-antiporter (Na^+ dependent) [4]. This model was reinforced by studies performed in the presence of the Na^+/H^+-antiporter inhibitor, 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) [5].
In order to establish whether the antiporter site was exclusive of *R.marinus* CpI we addressed ion translocation by the two most studied bacterial enzymes. We observed that *Ecoli* CpI also presents the antiporter activity, but that from *Pdenitrificans* does not. We proposed a correlation between the type of quinone used as substrate and the presence of the antiporter activity[6].
[1] Efremov etal(2010) *Nature 465, 441*.
[2] Hunte, C.etal(2010) *Science 329, 448*.
[3] Efremov and Sazanov(2011) *Nature 476, 414*.
[4] Batista, etal(2010) *BiochimBiophysActa 1797, 509*.
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[6] Batista and Pereira(2011) *BiochimBiophysActa 1807, 286*.

2915-Pos Board B685
Electron Tunneling in Respiratory Complex I
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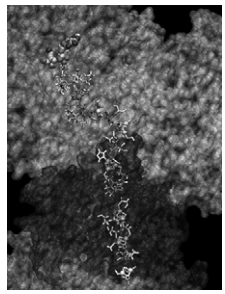
NADH:ubiquinone oxidoreductase (complex I) plays a central role in the respiratory electron transport chain by coupling the transfer of electrons from NADH to ubiquinone to proton pumping across the membrane. Until now, the atomistic details of electron transfer have remained unknown. In this study, electron tunneling along seven Fe/S clusters in complex I is examined in atomistic resolution by using the tunneling current theory and computer simulations [1]. Distinct electron tunneling pathways between neighboring Fe/S clusters are identified; the pathways primarily consist of two cysteine ligands and one additional key residue. The identified key residues are further characterized by sensitivity of electron transfer rates to their mutations, examined in simulations and their conservation among complex I homologues. Internal water between protein subunits is identified as an essential mediator enhancing drastically the overall electron transfer rate to achieve the physiologically significant value. With the water included, negative slope of the distance dependence of the electron transfer rates becomes close to a typical 1.4 in natural logarithm . The unusual electronic structure properties of Fe/S clusters in complex I explain their remarkable efficiency of electron transfer.



[1] T.Hayashi, A.A.Stuchebrukhov, *Proc. Natl. Acad. Sci. U.S.A.* **107**, 19157

2916-Pos Board B686
The Role of Acid Residues in Na^+ Uptake and Binding in Na^+-NQR from Vibrio Cholerae
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The Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) is the gateway for electrons into the respiratory chain of *Vibrio cholerae* and many other pathogenic bacteria. Na^+-NQR is unique among respiratory enzymes in that it pumps sodium rather than protons.
The character of Na^+ binding to the enzyme is crucial to understanding the coupling between Na^+ translocation and the redox reaction. We are using functional studies_steady state and transient kinetics and equilibrium binding_in combination with site-directed mutagenesis to investigate the interaction of Na^+ with Na^+-NQR. Recently, we have focused on conserved acid residues in membrane spanning regions as candidates for Na^+ binding site ligands. Here, we describe results on two of these residues, which are both involved in Na^+ uptake by the enzyme: NqrB-D397 and NqrE-E95.
Replacement of either residue by a neutral amino acid (Ala) results in a large increase in the apparent Km for Na^+. In the case of NqrB-D397, replacement by Glu or Cys, produced smaller changes in Km^{app}, indicating that the size and charge of the residue at this position both modulate Na^+ binding. Stopped-flow kinetic measurements show that mutations at both positions exert their effect specifically at one internal electron transfer step: 2Fe-2S center àFMN$_C$. These results are consistent with the earlier finding that this is the first Na^+ dependent electron transfer step in the enzyme reaction. The results are discussed will be discussed in the context of our current model of Na^+-NQR as a redox driven Na+ pump, that operates on the basis of kinetic rather than thermodynamic coupling.

2917-Pos Board B687
Suicide Inactivation in *Rhodobacter Sphaeroides* Cytochrome *c* Oxidase Lacking Subunit III Coincides with Release of Cu_B and Major Conformational Changes in Subunit I
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Cytochrome *c* oxidase from *Rhodobacter sphaeroides* shares homology with the three subunit core of the mitochondrial form. Subunits I (SI) and II contain the redox centers (Cu_A, heme *a*, and the binuclear center oxygen binding site, heme a_3 and Cu_B) of the enzyme. Subunit III (SIII) functions to inhibit turnover induced suicide inactivation by maintaining proton uptake into the D pathway and by stabilizing the heme a_3-Cu_B active site. Metal analysis of I/II oxidase, as determined by ICP-OES, shows that suicide inactivation leads to the release of one copper; EPR spectroscopy indicates that the missing copper is Cu_B. Limited proteolysis of wild-type and I/II oxidase using α-chymotrypsin showed no differences in the pattern of proteolytic digestion, however, SI of I/II oxidase was digested at a faster rate. MALDI-TOF and protein sequencing showed that the cleavage sites are localized to the N and C-termini of SI. Suicide-inactivated I/II oxidase exhibits a completely different digestion pattern, including the release of a peptide (AA 237-258 in SI; identified by MS-MS) that is located in a region above Cu_B in the enzyme. In an attempt to identify the oxidative reaction which leads to suicide inactivation, the two-electron reduced PM and three-electron reduced F intermediates were generated chemically in I/II oxidase. After incubation, the I/II oxidase was assayed for O_2 reduction activity and no inactivation was observed, suggesting that build up of strongly oxidizing chemical intermediates at the active site does not lead to suicide inactivation. These results suggest that suicide inactivation in I/II oxidase is triggered by a mechanism leading to Cu_B loss with concomitant conformation changes in SI.

2918-Pos Board B688
New Ligands of the Conserved Steroid Binding Site of Cytochrome c Oxidase
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Cytochrome *c* oxidase (C*c*O), the terminal enzyme in the electron transfer chain of mitochondria and many bacteria, requires the uptake of protons for activity via two pathways, the D-path and the K-path. Among the mutants of *Rhodobacter sphaeroides* (*Rs*) C*c*O that inhibit proton uptake in the K-path is E101A in subunit II, which removes a key carboxyl at the entrance to the pathway and decreases the activity of the purified enzyme over 20-fold. Micromolar levels of the bile acids cholate or deoxycholate were shown to stimulate E101A activity 10-fold, and crystals of wildtype *Rs*C*c*O grown in the presence of deoxycholate (Qin *et al*., *Biochemistry* 47:9931-9933, 2008) showed a single deoxycholate molecule bound close to E101 in the same location as a cholate molecule observed in bovine C*c*O crystal structure (PDB 1OCC). Evidence of protective effects of steroids against C*c*O inhibition in bilirubin neurotoxicity and in Alzheimers (Vaz *et al*., *J. Neurochem.* 112:56-65, 2010; Tillement *et al*., *Steroids* 71:725-735, 2006) suggest that this site could be physiologically relevant to regulation of C*c*O.
Here we use the *Rs*C*c*O mutant E101A as a sensitive assay to further investigate the nature of this conserved site. Activity assays show that certain other steroids (cholesterol hemisuccinate) and lipidic molecules (phytanic acid, arachidonic acid) and some detergents affect *Rs*C*c*O in a manner that indicates that these ligands also bind at the same site. The studies reveal a high degree of specificity and suggest possible physiological regulators. Crystallographic and computational approaches are underway to identify additional potential ligands and to determine the physiological significance of this conserved binding site. (NIH GM26916)

2919-Pos Board B689
A Kinetic Model of Proton Transport in a Multi-Redox Center Protein: Cytochrome c Oxidase
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Simulations of electrochemical measurements are presented, making use of a model system comprising cytochrome c oxidase (CcO) immobilized in a strict orientation on an electrode. This allows studying direct electron transfer (ET) to